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P. Fleckman and B.A. Dale, University of Washington, Department of Medicine, Division of Dermatology, Seattle, Wa./USA.

ICHTHYOSIS AND PROFILAGGRIN SYNTHESIS

The Ichthyoses are a heterogeneous group of inherited skin diseases, clinically manifest by scaling. Alterations in epidermal structural proteins and in lipid biosynthesis have been suggested as possible underlying defects in various ichthyoses. Studies in our laboratories have focused on the role of profilaggrin and filaggrin. Filaggrin is a keratin intermediate filament-associated protein derived from profilaggrin (proFG), a precursor expressed in the granular layer. ProFG is enzymatically processed to filaggrin, which aggregates keratin filaments in the lower stratum corneum and is then proteolyzed to component amino acids which act as humectants.

In types I and II Harlequin Ichthyosis proFG is expressed but not processed to filaggrin, while in type III, the protein is not expressed. In CHILD syndrome, proFG expression along with that of K1 and K10 is decreased. Exaggerated proFG protein expression is seen in Lamellar Ichthyosis and in BCIE. In X-linked Ichthyosis, a disorder with a known lipid defect, proFG is normal.

In Ichthyosis Vulgaris (IV) the granular layer is attenuated and keratohyalin granules (KHG) are abnormal. ProFG protein expression is markedly decreased, as shown by immunohistochemistry, immunoelectron microscopy, and Western blot, and proFG mRNA is decreased by in situ hybridization. Keratinocytes cultured from the epidermis of individuals with IV have decreased and abnormal appearing KHG and proFG protein and mRNA are barely detectable, while expression of K1/K10 and loricrin, also associated with differentiation, is normal.

Thus, the ichthyoses offer excellent models for understanding normal and pathologic epidermal differentiation. Abnormalities in proFG expression may offer clues to processes leading to these diseases.

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E. Epstein Jr., University of California, Department of Dermatology, San Francisco/Ca., USA.

EPIDERMOLYSIS BULLOSA SIMPLEX

Epidermolysis bullosa simplex (EBS) is characterized by basal keratinocyte fragility and consequent formation of blisters that, unlike deeper blisters, heal without scarring. Nearly always it is inherited as an autosomal dominant disease with complete penetrance.

During the past year we and several other groups have presented evidence indicating that this cellular fragility is due to mutations of genes encoding keratins that are expressed in the basal cells. This evidence is of three types.

First, genetic linkage analysis has mapped the EBS gene in a half-dozen families to chromosomes 12q or 17q at the sites to which keratin genes have been mapped. Second, point mutations have been identified in four families. Three of these are in keratin 14 and one is in keratin 5. The three families with the more severe Dowling-Meara subtype of EBS each have mutations in the conserved regions at the ends of the helical region; the one family with the Koebner subtype of EBS has a Leu to Pro mutation in the middle of the helix. Third, transgenic mice with mutant human keratin genes express a phenotype similar to that of human EBS.

These findings are among the early fruits of the application to human skin disease of the strategies and techniques of modern molecular genetics. Similar strategies have given evidence very recently that mutations of genes encoding keratins expressed in suprabasal keratinocytes underlie epidermolytic hyperkeratosis.

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A.M. Christiano, L. Chung-Honet, D.S. Greenspan*, A. Hovnanian, R.G. Knowlton, M.-L. Chu and J. Uitto, Jefferson Medical College, Philadelphia and *University of Wisconsin, Madison, USA and INSERM, Creteil, France.

CLONING AND CHARACTERIZATION OF HUMAN TYPE VII COLLAGEN, THE CANDIDATE GENE IN THE DYSTROPHIC FORMS OF EPIDERMOLYSIS BULLOSA

We recently demonstrated strong genetic linkage between the dystrophic forms of epidermolysis bullosa and the gene for human type VII collagen (COL7A1) on chromosome 3p21. To facilitate elucidation of the underlying mutations in these patients, we initiated extensive cloning of the corresponding gene and cDNA. In addition to our recently reported cloning of a human type VII collagen cDNA (PNAS 88, 6931, 1991), we have screened human epidermal keratinocyte, placenta, WISH and skin fibroblast cDNA libraries for clones coding for the α1(VII) chain. At this point, we have elucidated 8.7 kb of the ~9 kb mRNA. Deduced amino acid sequences revealed that the α1(VII) chain consists of a central collagenous domain characterized by repeating Gly-X-Y sequences which contain 22 imperfections, including a 39-amino acid non-collagenous "hinge" region. Sequences which encode the collagenous domain are flanked on the 5' side by >3.4 kb which encode a non-collagenous domain (NC-1), and on the 3' side by ~0.5 kb which encode a non-collagenous NC-2 domain. Detailed characterization of NC-1 revealed two high-frequency exonic polymorphisms which can be detected by PCR. The chimeric organization of NC-1 revealed modules with homology to cartilage matrix protein (CMP), nine consecutive fibronectin type III domains, and the A domain of von Willebrand factor. The cysteine-rich NC-2 domain contained a region with similarity to a Kunitz module found in α3(VI) collagen, among other proteins. Recent characterization of the human gene for type VII collagen has revealed over 100 exons in a compact gene of ~25 kb. Complete understanding of both the gene and cDNA for human type VII collagen will facilitate the identification of mutations in patients with the dystrophic forms of EB.

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M. Blumenberg, C.-K. Jiang, M. Tomic-Canic, T. Magnaldo and I.M. Freedberg, New York University Medical Center, Ronald O. Perelman Department of Dermatology, New York, USA.

REGULATION OF KERATIN GENE EXPRESSION BY HORMONES, VITAMINS AND GROWTH FACTORS

Keratins, the principal structural proteins of epidermis, are also markers of keratinocyte function. K#1 and K#10 mark the differentiated state, K#5 and K#14 the basal cells and K#6 and K#16 the activated cells.

Among the most important biological and therapeutic modulators of keratinization are hormones and vitamins, such as retinoids and corticosteroids, which act through their nuclear receptors. To understand the effects of the receptors on gene expression in epidermis, we have prepared DNA constructs in which promoters for the following keratin genes drive expression of the CAT reporter gene: K#3, K#5, K#6, K#10, K#14, K#16, K#17 and K#19. The constructs were co-transfected with vectors expressing nuclear receptors for retinoids, thyroid hormone, vitamin D3, estrogen and progesterone.

Thyroid hormone uniformly inhibited keratin gene transcription. Retinoic acid induced keratins K#8 and K#18, but suppressed all others. Vitamin D3 had no direct effect, estrogen upregulated keratins K#8 and K#18, whereas progesterone regulated the basal layer specific keratins. The regulatory elements were identified using in vitro mutagenesis. Thus, hormones and vitamins superimpose quantitative regulation of the levels of keratin gene transcription onto the qualitative, cell-type specific regulatory mechanisms.

Because peptide growth factors affect keratinocyte function, we hypothesized that they also could regulate transcription of keratin genes. Keratin promoter constructs were transfected into primary cultures of keratinocytes in the presence or absence of epidermal growth factor (EGF). While transcription of all transfected promoters was increased in the presence of EGF, the K#6 and K#16 keratin promoters were specifically induced ten fold or more. We have localized the EGF-responsive elements to 20 bp segments. We conclude that EGF alters the phenotype of keratinocytes by modifying the nuclear transcriptional machinery and inducing markers specific for activated keratinocytes.

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H. de Thé, C. Lavau, C. Chomienne*, A. Marchio, L. Degos* and A. Dejean, UREG, Institut Pasteur and *Hôpital St. Louis, Paris, France.

THE t(15,17) TRANSLOCATION OF ACUTE PROMYELOCYTIC LEUKEMIA GENERATES A FUNCTIONALLY ALTERED RETINOIC ACID RECEPTOR

Retinoic acid (RA) is a vitamin A derivative with major effects on differentiation and morphogenesis. Three retinoic acid receptors (RAR α,ß,γ) that belong to the nuclear hormone receptor superfamily have been described. RARß was originally identified at an hepatitis B virus integration site in a hepatocarcinoma, suggesting a link between altered receptors and transformation.

A specific translocation t(15,17) has been reported in every patient with acute promyelocytic leukemia (APL). Those patients achieve complete remissions when treated with oral all trans RA. This spectacular effect of the drug, together with the location of the RARα gene close to the breakpoint, suggested a (paradoxical) link between the two phenomenons.

An APL-derived cell-line (NB4) was used to clone this translocation and show that it fuses RARα to a new gene: PML. PCR analysis demonstrate the presence of a PML/RARα fusion mRNA in every patient, indicating that this genetic alteration is an APL hallmark.

The PML gene product displays a C3HC4 zinc finger motif common to several DNA-binding proteins and a leucine zipper; PML could encode a transcription factor. Hybrid cDNAs were isolated from the NB4 cell-line and shown to encode proteins containing the amino terminus of PML (including the zinc fingers and leucine zipper) fused to the B or to E regions of RARα (containing the DNA and hormone binding domains). This chimaeric transcription factor could interfere with either RAR or PML pathways. Transient expression studies show that PML/RARα is functionally altered when compared to RARα. Moreover PML/RARα can block RA-induced differentiation in some model systems. While interference with PML or RAR pathways could account for differentiation arrest and transformation, the spectacular effect of pharmacological doses of RA is poorly understood at present.

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J. Uitto, Thomas Jefferson University, Jefferson Medical College, Departments of Dermatology, Biochemistry and Molecular Biology, Philadelphia, PA./USA.

REGULATION OF COLLAGEN AND ELASTIN GENE EXPRESSION

Collagen and elastic fibers are the major extracellular matrix components of the dermis, providing physiologic tensile properties and resilience to the skin. Human skin contains at least

10 different collagen types which have a topographically compartmentalized tissue distribution. Cloning of different collagen genes has provided probes to study the regulation of collagen gene expression. A variety of cytokines, growth factors and hormones can alter the expression of collagen genes at the transcriptional level. These effects are mediated by a variety of cellular factors, including AP-1, AP-2 and NF-1, which bind to the corresponding *cis*-elements in the promoter region of the collagen genes. This presentation discusses the regulation of collagen gene expression in the context of fibrotic skin diseases.

Elastic fibers form a network which contributes to the elasticity of the skin. Characterization of the 5'-flanking region of the elastin gene has identified several *cis*-regulatory elements which include regions of enhancers and silencer activity, as well as putative elements responsive to a variety of effector molecules. Recent development of transgenic mice which express a human elastin promoter/CAT reporter gene construct, has revealed that the elements for tissue specific expression of the elastin gene reside within 5.2 kb of the 5'-flanking DNA. This animal model provides a system to study the transcriptional regulation of elastin gene expression by trans-acting cytokines and pharmacologic agents.

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J.J. Voorhees, University of Michigan, Department of Dermatology, Ann Arbor, Mi./ USA.

PHOTOAGING: MECHANISMS OF RETINOIC ACID-INDUCED IMPROVEMENT

Roughness, brown spots, and wrinkles are features of photodamage improved by topical all-*trans*-retinoic acid (TRA). Possible correlates of improved rough skin (i.e., smoother) due to TRA are compact and smooth stratum corneum (SC) and hygroscopic GAGs accumulating within epidermis and SC. In treated skin, K6 and K13 are focally expressed and K5/14 and K1/10 are expressed with correct location and intensity. TGase K, involucrin, loricin, and filaggrin are expressed with normal intensity but extend deeper into the thickened epidermis.

In photodamage, melanocytes are focally activated in brown spots. Such spots are lightened by TRA lasting > 6 months after RA is discontinued. Clinical lightening and light microscopic reduction of melanin are correlated ($r = -.53$, $p < .0001$). By electron microscopy, melanosomes and melanosome complexes in keratinocytes are markedly reduced. Thus, reduced melanin content is a probable cause of TRA's ability to lighten brown spots.

Wrinkle improvement by TRA appears related to dermal changes. TRA causes accumulation of epidermal and dermal TGF-β_1, a cytokine known to stimulate accumulation of collagens I and VII, both of which, by ultrastructural criteria, are increased by TRA in photodamage.

Improvement in photodamage is probably mediated by activation of RAR-γ and RXR-α in epidermis and RAR-β and RAR-γ in dermis. The amount of free RA available to activate RARs and RXRs may be regulated by high affinity cellular RA binding protein (CRABP-II) which is markedly increased by TRA. Free all-*trans*-RA is converted to 9 and 13-*cis*-RA, all 3 of which activate RARs whereas 9-*cis*-RA also activates RXR-α. Finally, TRA activity is greatly attenuated by major induction of cytochrome P-450 (type unkown), markedly reducing biological activity of TRA in photodamaged skin.

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M. Pfahl, La Jolla Cancer Research Foundation, La Jolla, Ca./USA.

THE STEROID/RETINOID HORMONE RECEPTOR SUPERFAMILY AND RETINOID RESPONSE PATHWAYS

The nuclear receptor superfamily represents one of the largest transcription factor families described today and includes receptors for all known active steroid hormones and the vitamin D3, as well as receptors for retinoic acid, thyroid hormones and peroxisome proliferator substances. In addition, many "orphan" receptors have been identified for which specific ligands have not yet been defined. The steroid hormone receptors and the retinoic acid receptors represent the two largest subfamilies of the superfamily, each comprising six individual receptors encoded by distinct genes. This paper focussed mainly on the mechanisms of action of retinoic acid receptors. Although it was originally assumed that all nuclear receptors function via the same basic mechanisms, it has become apparent now that the retinoic acid receptors operate quite differently than originally assumed. One of the RA receptor types, the retinoid X receptors (RXR) is already found in insects (in contrast to the other mammalian ligand binding receptors) and plays a central role in regulating the activity of several receptors, including thyroid hormone receptors (TRs), vitamin D3 receptors (VDR) and peroxisome proliferator activated receptors (PPAR). Contrary to earlier assumptions, the classical RA receptors (RARs) do not bind effectively to DNA and require auxiliary proteins for effective DNA interaction. The RXRs are such auxiliary receptors for RARs, dramatically increasing the affinity of RARs for specific DNA sites by heterodimer formation. Similarly RXRs function as auxiliary receptors for TRs, VDRs and PPARs. Thus, RXRs control a number of hormonal pathways through heterodimer formation. While RARs are activated by all-trans as well as 9-cis-RA, it was found that RXRs bind only 9-cis-RA with high affinity. This raised the possibility that the 9-cis-RA isomer could control specific response pathways. We have now obtained evidence that in the presence of 9-cis-RA, RXRs can form homodimers that activate specific genes. In contrast RARs do not form homodimers in the presence of 9-cis-RA (or all-trans-RA). Thus, RXRs have a central role in regulating distinct retinoid pathways through heterodimer formation with RAR and other hormonal receptors, while in the presence of 9-cis-RA, they can function independently by forming homodimers. The elucidation of the different retinoid signalling pathways should allow a more rational design of effective retinoid therapeutics while undesired side effects may be preventable.

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K. Kragballe, University of Aarhus, Marselisborg Hospital, Department of Dermatology, Aarhus, Denmark.

THE EFFICACY AND TOLERABILITY OF TOPICAL CALCIPOTRIOL IN PSORIASIS VULGARIS

Calcipotriol is a new synthetic vitamin D analogue developed for the topical treatment of psoriasis. Similar to the natural bioactive form of vitamin D3, 1,25-dihydroxyvitamin D3, calcipotriol binds to the intracellular vitamin D receptor and induces a number of biological activities such as inhibition of cell proliferation, induction of cell differentiation and modulation of T lymphocyte activities. In contrast calcipotriol is 100-200 times less potent in causing increased serum and urine calcium levels. This pharmacological profile makes calcipotriol an ideal candidate for the topical treatment of psoriasis. In dose-finding studies a concentration of 50 mikrogram/gram has been found to be optimal for the treatment of psoriasis vulgaris. Furthermore, treatment with calcipotriol ointment 50 mikgrogram/gram is slightly more effective than the treatment with betamethasone valerate ointment 0.1 % and short-contract dithranol therapy.

After stopping calcipotriol treatment, psoriasis may recur. If the treatment is maintained, it is possible to maintain the induced improvement. Furthermore, addition of UVB-light therapy can increase the anti-psoriatic effect induced by calcipotriol. Treatment with calcipotriol ointment can cause skin irritation, particularly in the face. Therefore, the face should not be treated. If used in amounts up to 100 gram per week, no changes were observed in any of the biochemical markers of bone and calcium metabolism. It is concluded that calcipotriol is an effective and well-tolerated drug for the topical treatment of psoriasis. It should be considered one of the first line drugs for the treatment of this disease.

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C. Gerst, M.-C. Lenoir-Viale, M. Darmon and **B.A. Bernard**, Centre International de Recherches Dermatologiques (CIRD) Galderma, Cell Biology Department, Sophia Antipolis, Valbonne, France.

RETINOIC ACID RESPONSIVE ELEMENT IN THE LONG CONTROL REGION OF HUMAN PAPILLOMAVIRUS

We previously reported (1) that addition of retinoic acid (RA) to normal human keratinocytes grown in medium containing delipidized serum resulted in the activation of transcription from the construct pH18CAT, containing the HPV18 long control region (HPV18-LCR) cloned upstream of the CAT reporter gene. We show here that in HeLa cells, the pH18CAT construct is transcriptionally down-regulated by RA when either RARα, RARβ or RARγl are cotransfected. We have localized a possible cis-acting RA-responsive element in the HPV18-E6 promoter region. Direct interaction of RARs with this sequence was demonstrated by gel-shift experiments. Moreover, this sequence confers RA-responsiveness to the herpes virus thymidine kinase (TK) promoter. This sequence contains the direct repeat motif AGTTCATGTTAAGGGTA similar to that identified as RA-responsive elements (RAREs) in the promoter region of the RARβ gene and other RA-responsive genes.

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L. Poellinger, A. Wilhelmsson, I. Pongratz, M. Whitelaw and A. Berghard*, Karolinska Institute, Department of Medical Nutrition and *Center for Biotechnology, Huddinge Hospital F-60, Novum, Huddinge, Sweden.

THE DIOXIN RECEPTOR: A LIGAND-ACTIVATED GENE REGULATORY PROTEIN

Dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin) induces transcription of a number of genes encoding drug metabolizing enzymes such as glutathione S-transferase Ya and cytochrome P-450IA1. Signal transduction by dioxin is mediated by the intracellular dioxin receptor which, in its ligand-activated state, interacts with dioxin-responsive positive DNA control elements near the regulated promoters. The inactive receptor form is associated with an inhibitory protein (hsp90). Hsp90 plays dual roles in the modulation of functional receptor activites: it is critical for a dioxin-responsive conformation in that it is required for formation of a stable ligand-receptor complex; and it inhibits the DNA-binding activity of the receptor. Moreover, receptor activity appears to be modulated by protein kinase C-dependent phosphorylation, and by heterodimerization of the 100 kD dioxin-binding subunit with an 85 kD non ligand-binding protein which may be related to the recently cloned helix-loop-helix factor, Arnt, which controls nuclear translocation of the dioxin receptor.

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A. Aström, U. Pettersson and J.J. Voorhees, University of Michigan, Department of Dermatology, Ann Arbor, Mi./USA.

CLONING OF THE HUMAN CELLULAR RETINOIC ACID-BINDING PROTEIN II (CRABP-II) GENE: TRANSCRIPTIONAL REGULATION BY RETINOIC ACID

The human CRABP-I and CRABP-II cDNAs have recently been cloned from human skin. Furthermore, retinoic acid (RA) causes a striking induction of CRABP-II but not CRABP-I mRNA in skin in vivo and in skin fibroblasts in vitro. To further dissect the mechanism for retinoic acid induction of CRABP-II, we cloned the human gene. A human genomic library was screened using the CRABP-II cDNA as a probe. A clone approximately 16.5 kb in size was isolated, and by restriction mapping and sequencing was found to contain the entire CRABP-II gene. The gene is approximately 6 kb in size and divided into 4 exons. The upstream region of the gene contains a TATA box and potential binding sites for SP1, AP2 and *Krox*-24, as well as a direct repeat with homology to the retinoic acid responsive element (RARE) found in the RAR-β_2 gene.

The CRAPB-II mRNA was rapidly induced within 2-6 h in cultured human skin fibroblasts by retinoic acid, reaching a plateau after 6 h of treatment. However, removal of retinoic acid from the medium after 12 h caused a sharp decline of CRABP-II mRNA levels. The rapid increase of CRABP-II message was mainly due to an increased rate of transcription as determined by nuclear run-on experiments. Increased transcription could be detected as early as 1 h after addition of RA, peaked at 2 h and returned to basal levels within 6 h. On-going protein synthesis was required for this transient increase of transcription, since the induction was blocked by cycloheximide. These data suggest that the human CRABP-II gene is transcriptionally regulated by a newly synthesized regulatory protein. However, once CRABP-II mRNA is produced, message stabilization may be a means by which elevated CRABP-II mRNA is maintained.

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U. Reichert, J.M. Bernardon, B. Charpentier, B. Martin, B. A. Bernard, D. Asselineau, S. Michel, M. Régnier, W. R. Pilgrim, Y. M. Darmon and B. Shroot, Centre International de Recherches Dermatologiques (CIRD) Galderma, Sophia Antipolis, Valbonne, France.

SYNTHETIC RETINOIDS - RECEPTOR SELECTIVITY AND BIOLOGICAL ACTIVITY.

Retinoids constitute one of the most promising groups of drugs in dermatology today, although they exhibit a series of toxic

effects. The recent discovery of three distinct nuclear retinoic acid receptors (RARα, RARβ, RARγ) offers an appealing rationale for an approach to improve the benefit/risk ratio by the design of receptor selective compounds. With this objective in mind we evaluated a series of derivatives of 6-aryl-β-naphthoic acid, stable retinoic acid analogues, for (i) their in vitro affinity to recombinant human receptors RARα, RARβ, and RARγ, (ii) their transactivating potential in HeLa cells cotransfected with the appropriate RAR expression vectors and a TRE3-tk-CAT reporter plasmid, (iii) the induction of plasminogen activator in the murine teratocarcinoma cell line F9, and (iv) the repression of plasma membrane-associated transglutaminase in cultured human keratinocytes.

We found that variation of substituents on the aryl ring have little effect on binding to RARα but that opening of the naphthoic acid ring, which results in benzoic acid derivatives, improves the affinity to RARα. Reduced lipophilicity in the central region of the molecule, and particularly the introduction of functions with hydrogen bonding properties potentiates RARα binding and, when combined with certain aryl substituents, results in RARα selectivity. On the other hand, binding to RARβ and RARγ is strongly influenced by para (but not meta)-substituents of the aryl ring: hydrogen bond acceptors in this position increase RARβ selectivity, whilst hydrogen bond donors promote RARγ selectivity.

Transcriptional activation is more sensitive than in vitro binding, but results essentially in the same receptor selectivity profile, whereas the correlation between receptor selectivity and biological activity in genetically 'non-manipulated' cellular systems (F9, keratinocytes) is poor. Nevertheless, preliminary results obtained with a reconstructed skin model indicate that receptor selective retinoids possess discriminating properties, which influence epidermal morphology and differentiation.

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O. Danos, Institut Pasteur, Laboratoire Rétrovirus et Transfert Génétique, Paris, France.

VIRAL VECTORS FOR GENE THERAPY

More than twenty families of viruses are known to infect animal and human cells. Each has evolved a unique strategy for selecting and colonizing cells where viral multiplication can occur. The genetic material of viruses can be manipulated as cloned genes and cDNA and a wealth of knowledge has accumulated concerning the molecular mechanisms of viruses growth and interaction with the infected cell. As a consequence, it is possible in many cases to design and assemble recombinant viruses, where foreign genes are inserted into the viral genome and transported to the cell through the infectious process. The major applications of viral vectors are: a) the efficient transfer and expression of genes in tissue culture, b) the design of vaccines using recombinant attenuated viruses displaying antigenic epitopes from various pathogens, and c) the in vivo delivery of therapeutic genes.

The later application requires a viral vector able to efficiently transfer its genetic material to a variety of cell types, without affecting their physiological properties. Transferred genes must be stably maintained into the target cell, either as an integrated structure linked to the genome or as an episome. The vector design must allow for the sustained expression of the implanted therapeutic gene over long periods of time. Finally, the potential risks associated with the vector system (oncogenicity, viral disease) must be documented.

Three human DNA viruses (Herpes Simplex Virus, Adenovirus and Adeno-Associated Virus) and a murine retrovirus (Mouse Leukemia Virus) are currently studied as vectors for gene therapy. Until now, only retroviral vectors have been used in clinical trials.

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L. Degos, S. Castaigne, P. Fenaux* and C. Chomienne**, Hôpital St. Louis, Service Clinique des Maladies du Sang, Paris, *Hôpital Claude Huriez, Service des Maladies du Sang, Lille and **Hôpital St. Louis, Institut Universitaire, Paris, France.

ALL TRANS RETINOIC ACID: A TARGETTING DRUG FOR THE DIFFERENTIATION OF ACUTE PROMYELOCYTIC LEUKEMIA

All-trans retinoic acid (ATRA) is able to specifically differentiate acute promyelocytic leukemic cells (APL) in short term culture (Chomienne et al., Blood 1990). Patients with APL achieved complete remission within 1 to 3 months by a progressive maturation of leukemic cells (Huang et al., Blood 1988; Degos et al., Lancet 1990). The advantages of this differentiation therapy is the rapid disappearance of the bleeding disorder and the absence of aplastic phase avoiding the early deaths occuring in 15 to 30 % of patients with conventional chemotherapy. However, relapses occur when ATRA alone is maintained. For this reason, a chemotherapy is added after complete remission obtained by ATRA. A pilot study on 27 patients was proposed with the sequential combination of ATRA and chemotherapy leading to 70 % of actuarial event free survival (83 % actuarial disease free survival) at 18 months. A European trial randomizes conventional therapy to the sequential ATRA-chemotherapy protocol (70 patients included).

Retinoic acid receptor (RARα) is rearranged by the specific translocation t(15;17) of APL (de Thé et al., Nature 1990); a PCR technique was developped (Castaigne et al., Blood, in press) in order to ensure the diagnosis and to follow the minimal residual disease. Transfection experiments of the chimaeric gene in granulocytic cells (HL60) specifically inhibits the in vitro differentiation induced by retinoic acid (Farzinet et al., Nature, in press). The arrest of maturation of granulocytic lineage could be one of the major step of the leukemogenesis. ATRA is able to revert the arrest of maturation may be through a modulation of the expression (increased) of the normal allele of RAR (Chomienne et al., J.Clin.Oncol. 1991), which could overpass the impairment induced by the chimaeric protein on target responsive elements. One of the steps of the repair is the modulation of programmed cell death (PCD). Bcl-2, a gene involved in the PCD, is modulated in in vitro studies, arguing for the engagement of the cell in the natural death (Chomienne et al., in preparation). The beneficial effect of "differentiation therapy" probably is due to the induction of the natural death of the malignant cell.

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R.M. Lavker, C. Wilson, G. Cotsarelis and T.-T. Sun*, University of Pennsylvania, School of Medicine, Department of Dermatology, Philadelphia and *New York University Medical Center, Department of Dermatology and Pharmacology, New York, USA.

THE TELOGEN FOLLICLE: A MODEL FOR STUDYING HAIR GROWTH

The hair follicle is an epidermal derivative which independently undergoes a regular cycle of growth (anagen), involution (catagen) and rest (telogen). During the growing phase a new outer root sheath is formed and the matrix keratinocytes located in the bulb area proliferate rapidly. At the end of anagen, matrix keratinocytes abruptly cease proliferating and these cells necrose so that the lower follicle involutes. During this time the mesenchymal cells of the follicular papilla become condensed and are positioned at the bottom of the permanent portion of the hair follicle, and remains there during the telogen stage. Eventually a new growing phase occurs, and the cycle is repeated.

We have found that a subpopulation of relatively primitive, slow-cycling cells, which can be stimulated to proliferate in response to wounding (features consistent with stem cells), are located in the upper portion of the follicle in a region known as the "bulge". We proposed that these hair follicle stem cells, upon division, would give rise to a population of transient-amplifying (TA) cells which would differentiate into outer root sheath and matrix keratinocytes (Cell 61, 1329, 1990). This theory is in contrast to the widely-held belief that the germinative center of the hair follicle is in the anagen bulb, and that the follicular stem cells are located in the matrix keratinocytes.

A central tenant of our hypothesis is that at the beginning of anagen, the normally slow-cycling stem cells of the bulge area would be "activated" by the abutting follicular papilla cells and proliferate giving rise to a population of TA cells, which form the new downgrowth. Furthermore, we postulated that the bulge cells would return to their quiescent state during mid- to full-anagen. Using morphological and cell kinetic techniques we provide experimental evidence to show that in early anagen mouse skin, bulge cells indeed undergo transient proliferation during early anagen, giving rise to the highly proliferative matrix keratinocytes of the new hair follicle. We also report that during the resting phase, the bulge cells can be stimulated to proliferate in response to anagen follicle. These observations lend strong support to our bulge activation hypothesis and support the suggestion that bulge cells are the origin of the lower follicle in anagen. They also establish the usefulness of the resting phase of the hair cycle as a model to study the effects of various growth stimuli on hair follicle formation.

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P.J.A. Davies, V. Gentile, M. Saydak, V. Thomazy*, D. Gil** and R. Chandraratna**, University of Texas Medical School, Department of Pharmacology, Houston, Texas/USA, *University School of Medicine, Department of Pathology, Debrecen, Hungary and **Allergan Inc., Irvine, Ca./USA.

RETINOID RECEPTOR-REQUIRED EXPRESSION OF TISSUE TRANSGLUTAMINASE

The expression of Tissue Transglutaminase (TTg) is under complex control, operating at a very low level in most undifferentiated and proliferating cells and increasing dramatically as cells enter into states of terminal differentiation, senescence and death. Previous studies from our laboratory have identified retinoids as acute, specific and direct regulators of TTg gene expression. In the studies to be reported here we have used a combination of pharmacologic and transfection approaches to establish the role of specific retinoid receptors in the induction of the enzyme. Our results demonstrate that the expression of this enzyme is regulated both by retinoid acid receptors (RAR's) and receptors for 9-cis-retinoic acid (RXR's). To investigate the biological role of retinoid-regulated TTg expression we have compared the localization of the enzyme with the expression of specific RAR's in developing chick limb buds. The results demonstrated TTg expression co-localized to sites with both RAR-β or RAR-γ expression. These studies suggest that the expression of TTg can be linked to the activation of multiple retinoid receptors.

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M. Vasseur, Université Paris VII, Hall de Biotechnologie, Paris, France.

ANTISENSE APPROACHES IN DERMATOLOGY

Antisense technology, based on the design of antisense compounds complementary to an RNA or a DNA sequence critical to a disease process, offers the potential to revolutionize the pharmaceutical industry. Antisense represents a rational drug design approach, in which the genetic code of the disease dictates the structure of the antisense drug.

A therapeutic approach targeted to mRNA by means of antisense oligonucleotides or ribozymes is a wide ranging technology. Once established, this technology could be potentially applied to any disease in which the expression of a known responsible gene is involved. However, the development of effective delivery methods will be of vital importance to the market growth for antisense molecules.

It is obvious that topical delivery of dermatological drugs allows to circumvent most of the difficulties of targeted delivery. For antisense oligonucleotides and ribozymes, topical applications for dermatological diseases is a preferred way for an easy and targeted delivery. Apart from the viral infections such as Herpes or Papilloma, many other dermatological diseases including inflammatory diseases as atopical dermatitis or lupus, diseases of the keratinization process such as ichthyosis and psoriasis, neoplasic diseases such as melanoma or cutaneous T lymphoma, represent potential targets for an antisense therapeutic approach.

The dermatological drugs and the cosmetic market is wide and is certainly one of the most promising for the use of antisense drugs.

Dermatological pathologies, as well as ex-vivo treatments of hematological diseases, will be one of the first applications of antisense molecules. Current investigations, clinical trials in progress, patent and regulatory issues will be discussed.

P1

B. Algermissen, K. Hamann*, E. Ruch, F.W. Bauer and B.M. Czarnetzki, Hoffmann-La Roche , Department of Dermatology, Basel, Switzerland and *UKRV, The Free University of Berlin, Department of Dermatology, Berlin, Germany.

DISTRIBUTION OF MC_T, MC_C and MC_{TC} IN PSORIASIS, ATOPIC DERMATITIS AND LICHEN PLANUS

Human skin mast cells contain a number of preformed mediators which are released after immunological and non-immunological stimuli. Among them are two serine proteases, tryptase and chymase, which represent over 50 % of the total protein of the mast cell granules. Due to the proteolytic effect on protein substrates important in the elicitation of an inflammatory response (Il1, VIP), the release of these proteases may play a role in physiological and pathophysiological conditions.

We investigated the distribution of tryptase-(MC_T), chymase-(MC_C) and tryptase/chymase-containing (MC_{TC}) mast cells in involved skin from psoriasis, atopic dermatitis and lichen planus by enzymhistochemical techniques using specific substrates for tryptase and chymase.

In fresh frozen biopsies of involved skin from psoriasis (9/13) and lichen planus (6/6), we observed a high increase of MC_T in stratum papillare but not in the stratum reticulare. In psoriasis and lichen planus the MC_T were particularly prominent beneath the epidermis in close juxtaposition to the stratum basale. In two cases of lichen planus the MC_T could be identified in the stratum basale.

In atopic dermatitis (8/8) MC_T were also increased near the epidermal boundary and in addition, around the skin appendages such as hair follicles, blood vessels and sweat glands. While MC_C were occasionally identified in involved skin from psoriasis and atopic dermatitis, the MC_{TC} were found in small numbers in the stratum papillare and particularly in the stratum reticulare.

Our results show not only increased numbers of mast cells in involved compared to uninvolved skin but a shift to MCT which are extremely rare in normal skin. These MCT may be the result of inflammatory events and may play a role in the initiation and maintenance of inflammatory diseases. Tryptase, the main protease in these mast cells, may be involved.

P2

D. Asselineau and M. Darmon, Centre International de Recherches Dermatologiques (CIRD) Galderma, Sophia Antipolis, Valbonne, France.

ORAL METAPLASIA OF ADULT HUMAN EPIDERMAL KERATINOCYTES GROWN IN VITRO IN THE PRESENCE OF RETINOIC ACID

A striking effect of retinoic acid (RA) is its ability to alter cell fate during development. The mucous metaplasia produced by treating chick embryo skin with RA is a classical example of this property. It has so far been impossible to reproduce with adult keratinocytes grown in vitro such a metaplasia, although RA has been shown to block terminal epidermal differentiation, to induce an increased synthesis of mucopolysaccharides, and to induce markers of non-keratinized epithelia such as K19 and K13 keratins. When adult human keratinocytes are grown on dermal lattices at the surface of the culture medium, they are able to form a normal keratinized epidermis. But, when excess RA is added to the culture medium, a stratified non-keratinized (parakeratotic) epithelium is formed. The distribution of tissue- and differentiation-stage specific markers in this epithelium shows that it has close resemblance with the oral epithelium. Moreover, when this tissue is transferred into a normal medium (no RA added), a new epithelium is formed beneath the "old" one at the expense of basal cells, and this epithelium has an epidermal orthokeratinized phenotype, whereas the "old" epithelium remains unchanged. These observations show that the reversibility of the changes produced by RA are only apparent, since the metaplastic tissue remains unchanged and the new tissue is derived from still uncommitted cells. Altogether, our studies suggest that adult keratinocytes treated in vitro by RA, although unable to transform into mucous cells, undergo a metaplasia into a wet stratified epithelium closely resembling oral epithelia.

P3

S. Barlati, L. Moro, N. Zoppi and M. Colombi, University of Brescia, Division of Biology and Genetics, Department of Biomedical Sciences and Biotechnologies, Brescia, Italy.

CORRECTION OF THE DEFECTIVE FIBRONECTIN EXTRACELLULAR MATRIX OF EHLERS-DANLOS SYNDROME SKIN FIBROBLASTS BY DEXAMETHASONE TREATMENT

The Ehlers-Danlos syndrome (EDS) constitutes a heterogenous group of heritable connective tissue disorders which can be distinguished in 11 different types on the basis of clinical manifestations and pattern of inheritance.

We report that in vitro cultured skin fibroblasts from EDS type 1 to VIII lack an organized fibronectin (FN)-containing extracellular matrix (ECM); this defect can be corrected by cultivation of EDS cells over a feeder of control fibroblasts or by the addition into the culture media of FN purified from control fibroblasts (cellular FN: cFN) but not of FN obtained from plasma (plasma FN: pFN). cFN differs from pFN for the presence of higher levels of isotypes containing a region undergoing alternative splicing (i.e. EDA). Quantitative in situ hybridization shows that EDS cells mature reduced amount of EDA^+ FN mRNA if compared with control fibroblasts, thus suggesting a relationship between alternative splicing processes and the FN-ECM defect in EDS cells.

In vitro cultured skin fibroblasts derived from EDS type I to VII treated with dexamethasone (Dex) - a synthetic glucocorticoid known to stimulate the biosynthesis of FN in in vitro cultured cells - organize a FN-containing ECM. The ECM correction is related to the induction of FN mRNA in all cell types. These data suggest the possible use of Dex in the therapy of the most severe EDS cases.

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P4

S. Beninati and A. Abbruzzese*, II. University of Rome "Tor Vergata", Department of Biology, Rome and *University of Naples, Department of Biochemistry and Biophysics, Naples, Italy.

SPERMIDINE-MEDIATED CROSS-LINKING IN MAMMALIAN EPIDERMIS: A POTENTIAL MARKER FOR KERATINOCYTE DIFFERENTIATION

The N^1,N^8-bis(γ-glutamyl)spermidine cross-link occurs as a post-translational modification in several structural proteins. These cross-links are formed by calcium-required transglutaminases, which catalyze the formation of a covalent bond between the carboxyl group of the γ-carbon of peptide-bound glutamine residues and the primary amine groups of spermidine. The glutamyl-spermidine cross-link is resistant to enzymic and chemical attack and it is presumed that its biological role in the case of epidermis is a structural one. Recently this cross-link was found in proteins of the cornified envelope of cultured human epidermal keratinocytes. Significantly higher than normal frequency of this cross-link was observed in envelopes from afflicted areas of psoriatic patients. These chemically-resistant protein envelopes develop beneath the plasma membrane of cells in the stratum corneum during epidermal differentiation. Spermidine cross-link was located in the fraction insoluble to thiol-SDS extraction. The polymerization pathway of proteins by spermidine proceeds by two steps. Initially, a primary amino group reacts with a γ-glutamyl site of a protein, leading to the formation of a mono-(γ-glutamyl)spermidine. Finally, the remaining primary amine group is cross-linked to another γ-glutamyl site. This mechanism suggests that soluble acceptor substrates are present in the cytoplasm of the living cell layers of the epidermis. It was envisaged that during the course of epidermal differentiation, these proteins become increasingly cross-linked because of epidermal transglutaminase activity, resulting in a polymer which could only be solubilized by treatments that cleaved peptide bonds. Analysis of spermidine cross-links in malignant or virally transformed human keratinocytes shows a severe reduction of the level of this cross-link. Additionally studies are in progress to evaluate the usefulness of this post-translational modification of proteins as a marker in staging and biochemically characterizing abnormally keratinizing epidermal cell lines and epidermis.

P5

G. Borroni, A. Riccardi*, R. Rosso**, G. Vignati, C. Zaccone, G.P. Vignoli and G. Rabbiosi, University of Pavia, Department of Human and Hereditary Pathology, Institute of Dermatology, *Department of Internal Medicine II and **Institute of Pathology, IRCCS Policlinico S. Matteo, Pavia, Italy.

13-CIS-RETINOIC ACID IN REFRACTORY CUTANEOUS KI-1 LYMPHOMA

Increasing interest has recently been shown as regards the therapeutic effects of systemic retinoids on dermatological malignancies following successful results in T-cell erythroderma

and mycosis fungoides. A report on the response to 13-cis-Retinoic Acid in refractory Ki-1 lymphoma is given relating to a 34-year-old male patient in whom papular and nodular lesions which had been present for 2 years continued to relapse despite a variety of treatments (Re-PUVA, systemic steroids, electron beam therapy). The cutaneous necrotic nodules recurred after a few weeks partial remission with each therapy. Histopathological findings from the three skin biopsies taken revealed an unchanged pattern of malignant T-cell lymphoma with features common to pagetoid reticulosis, large anaplastic T-cell lymphoma and nodular mycosis fungoides. Immunophenotyping with a panel of T-cell and B-cell specific monoclonal antibodies gave a pattern of Ki-1 positive cutaneous T-large cell lymphoma. Complete remission with the disappearance of the nodules was obtained for a period of 10 weeks with a daily regimen of 60 milligrams of oral 13-cis Retinoic Acid (1 mg/kg/day) followed by sudden and total relapse during the therapy. Contrary to reports by other authors, the effect of 13-cis-Retinoic Acid was only temporary and, although this drug should be considered in the treatment of cutaneous Ki-1 lymphoma, further reports are clearly required before any picture can be built up of the appropriateness of this therapy.

P6

N. Boyera, D. Cavey, F. Delamadeleine, M. Bouclier, C. Hensby and B. Shroot, Centre International de Recherches Dermatologiques (CIRD) Galderma, Sophia Antipolis, Valbonne, France.

A NOVEL IN VITRO MODEL FOR THE STUDY OF HUMAN KERATINOCYTE/LEUCOCYTE INTERACTIONS UNDER AUTOLOGOUS CONDITIONS

Keratinocyte/leucocyte interactions have become an area of intense investigations in the last decade. However, only few convenient in vitro models are available at this time. Thus, we designed a novel in vitro system for autologous human keratinocyte/leucocyte co-culture. Non invasive epidermal cell sampling was achieved by using outer root sheath cells from hair follicles: after one passage, pure keratinocyte cultures (no Langerhans cells or melanocytes) were obtained. Co-culture experiments were performed on a Transwell system : keratinocytes were grown on the porous cupula, and then laid onto wells containing leucocytes. Alternatively, leukocytes could be added to the cupula when contact interactions between the two cell types have to be investigated.

Using this system, we demonstrated that PHA-P activated T lymphocytes (with 10% monocytes) in the lower compartment induced ICAM-1 and HLA-DR expression, and inhibited methyl-^{3}H-thymidine incorporation in normal human autologous keratinocytes cultured on the cupula. These changes were mediated by soluble factors (no cell contacts between keratinocytes and leucocytes) and required lymphocyte activation.

This is the first direct in vitro evidence for leucocyte-induced ICAM-1 and HLA-DR expression on keratinocytes. This system is a potential tool to study keratinocyte/leucocyte interactions : it is easy to perform, keratinocyte and leukocyte responses can be assessed separately (proliferation , surface antigen expression), experiments within a given donor can easily be reproduced, and this model lends itself to a vast range of different experimental conditions.

P7

B. Charpentier, J.M. Bernardon, C. Gatje, B. Martin, B.A. Bernard, Y.M. Darmon, W.R. Pilgrim and B. Shroot, Centre International de Recherches Dermatologiques (CIRD) Galderma, Sophia Antipolis, Valbonne, France.

NEW RETINOIDS WITH SELECTIVITY FOR BETA AND/OR GAMMA NUCLEAR RETINOIC ACID RECEPTORS

Retinoic Acid (RA) plays a fundamental role in cellular proliferation and differentiation. RA and a few synthetic analogues (retinoids) are currently used for the treatment of certain dermatological disorders, mainly acne and psoriasis. The discovery of several nuclear receptors for RA has engendered hopes that analogues with selective affinity for one of these receptors will possess a more tissue-specific activity than RA and therefore could be associated with reduced side effects. In previous work we have reported that biological activity of retinoids in F9 cells correlates with their affinity for nuclear receptors present in whole cells.

Here we report a further characterization of the family of compounds related to the new dermatological agent ADAPALENE (CD 271)*. Briefly, we have carried out structure/activity and structure/affinity studies of a series of retino-naphthoic acids by systematic variation of substituent groups on the aromatic ring which corresponds to the lipophilic region of RA. The binding affinities for isolated receptors and the in vitro biological potencies (differentiation of murine F9 teratocarcinoma cells) of all compounds are reported here and related to their molecular structures. Compounds exhibiting selectivity for either the Beta and/or Gamma retinoic acid receptor were identified (B. Martin et al., Skin Pharmacol, 1992, 5, 57-65). Relationships between in vitro differentiating potencies and binding affinities for each RAR are discussed.

* CD271: 6-[3-(1-Adamantyl)-4-methoxyphenyl]-2-naphthoic acid.

P8

M. David, M. Lapidoth, D. Ben-Amitay and V. Katznelson, Tel Aviv University, Beilinson Medical Center, Department of Dermatology, Sackler Faculty of Medicine, Petah Tiqva, Israel.

THE EFFECT OF COMBINED TREATMENT WITH PREDNISONE AND CYCLOSPORINE IN PEMPHIGUS VULGARIS - A COMPARATIVE OPEN STUDY

Sixteen hospitalized pemphigus vulgaris patients received combined treatment with cyclosporine and prednisone for 12 months. Cyclosporine was given orally twice daily, with the initial dose of 5 mg/kg/day adjusted to obtain plasma levels of 100-150 ng/L. Prednisone was given at an initial dose of 60-80 mg/day, with tapering in accordance with clinical improvement. Fifteen pemphigus patients receiving prednisone alone at an initial dose of 120 mg/day served as a comparative group. Thirteen of the 16 patients completed one year of follow-up. All 16 patients achieved clinical remission within 25 days or less. New blister formation ceased after a mean of 11.1 days of onset of treatment in the combined treatment group versus 20.5 days in the comparative group ($p=0.004$). Hospital stay was shorter in the combined treatment group (mean, 32.6 days) than in the comparative group (mean, 50.7 days; $p=0.003$). The mean total accumulative prednisone dosage during hospitalization and follow-up was 8853 mg in the combined treatment group and 12,977 mg in the comparative group ($p=0.008$). The most common side-effect in the 16 patients receiving combined treatment was fatigue (10 patients), followed by muscle and joint pain (9 patients), and hypertension and hypertrophic gingivitis (6 patients each). In view of our results it seems that combined use of prednisone and cyclosporine is more effective than prednisone alone for the treatment of pemphigus. In addition, this preliminary study indicates a corticosteroid-sparing effect of cyclosporine. A large double-blind, randomized study is needed to confirm these findings.

P9

R. Debets, J. Hegmans, E. Prens, R. Troost* and R. Brenner, Universital Hospital Dijkzigt and Erasmus University Rotterdam, Department of Immunology and *Department of Dermatology, Rotterdam, The Netherlands.

ALTERED IL-1 and IL-6 RELEASE BY PSORIATIC EPIDERMAL CELLS AND ITS RESTORATION BY TOPICAL CORTICOSTEROIDS

Dysregulation of IL-1 and increased levels of IL-6 have been observed in psoriatic lesions. However, the data on these cytokines are often seemingly contradictory due to the use of different types of skin samples and single assays for their detection. We determined the basal levels of immunoreactive as well as biologically active IL-1 and IL-6 released by epidermal cell (EC) suspensions of psoriatic and normal healthy skin. Freshly isolated, vital EC were cultured in low Ca^{2+} basal medium (10^6 cells/ml) for 24 hours after which supernatants were collected. The levels of both cytokines were measured in all samples using ELISA's and the D10 and B9 bio-assays in parallel. The results showed that IL-1β immunoreactivity was increased by more than 10 to 20 fold in 80 % of the psoriatic EC supernatants whereas the bioactivity was reduced. These findings are in agreement with reported data on IL-1 in skin homogenates and point to a possible involvement of an inhibitor for IL-1. The ELISA and bio-assay results for IL-6 correlated well. Both tests showed that IL-6 production by freshly isolated EC was elevated in 50 % of the patients. In vivo treatment for six weeks with topical corticosteroids restored the altered cytokine profiles. The abnormal patterns of IL-1β and IL-6 release by unstimulated psoriatic EC stress their primary involvement in psoriasis.

P10

M. Démarchez, P. Rocheton and J. Czernielewski, Centre International de Recherches Dermatologiques (CIRD) GALDERMA, Sophia Antipolis, Valbonne, France.

A MODEL OF CHRONIC ECZEMA IN THE HAIRLESS MOUSE

Most of the animal models of contact eczema mimic acute human pathology. In the present study, it is demonstrated that once daily topical applications of low dose of oxazolone (0.3%) on the dorsal skin of hairless mice induce an acute contact eczema which develops into a chronic state. After one week of treatment, the animals appeared to be sensitized to the hapten. Macroscopical and histological studies and measurements made with the MOP-Videoplan system at different stages during the treatment, ranging from one week to 11 weeks, showed that during the second week, an acute lesion develops which is characterised by extensive desquamation, erythema, spongiotic vesicles, and increase of epidermal and dermal thickness. At later stages, this acute lesion changes to a chronic state characterized by a decrease in desquamation and spongiotic vesicles, maintenance of erythema, hyperplasia, and dermal oedema, and presence of dermal infiltrates. Numeration of dermal mast cells indicated that their number began to increase during the third week and continued to increase constantly during the following weeks becoming 5 times higher than control at 11 weeks. Topical treatment with 2 NSAIDs (parfenac and piroxicam) or 3 steroids (hydrocortisone, Betamethasone-17-valerate (BMV), or dermoval) of the acute lesion, or of the chronic lesion, indicated that only the two steroids (BMV, or dermoval) were active on both types of established lesions. They reduced hyperplasia, dermal oedema and dermal infiltrates. In conclusion, the present data describe a model of chronic eczema in mouse which demonstrates numerous similarities with the human pathology, such as the mode of induction, numerous morphological characteristics, and its pharmacological response. As the mode of action of hapten, such as oxazolone is to form a complex with an epidermal protein and to induce an immune mediated response to this complex, it is proposed that such a model could also serve to mimic certain aspects of auto-immune diseases, such as atopic dermatitis or psoriasis.

P11

E. Demirpence*, M. Pons* and D. Gagne*,**; *INSERM U 58 and **Faculté de Pharmacie, Laboratoire de Biochimie, Montpellier, France.

USE OF BIOLUMINESCENT CELLULAR MODELS FOR STUDYING INTERACTIONS BETWEEN ESTROGENIC AND RETINOID ACTIVITIES

The antiestrogenic action may occur via different mechanisms. The first one, which is characteristic of classical antiestrogens (e.g. OH Tamoxifen) results from a competition with estradiol for binding the estrogen receptor (ER). The second one results from an inhibition of ER activity at its responsive element (ERE), for example by altering the amount of ER bound to the ERE or affecting its transcriptional activity.

With the aim of researching new antiestrogenic molecules, we have previously established the chimeric MVLN cell line which consists of stably transfected MCF-7 cells expressing the firefly luciferase in an estrogen-dependent manner. In such a model, an antiestrogenic effect, located at any step of the estrogenic action, may be evaluated. Thus, we showed that retinoic acid behaved as an antiestrogen and this effect, which did not interfere with the binding of estradiol to its receptor, appeared to be mediated by the second type of mechanism described above.

In order to investigate the possible interactions between different steroid activities and the retinoid one, we developed MRLN cells which consist of stably transfected MCF-7 cells expressing the firefly luciferase in a retinoic acid-dependent manner. We present here some results obtained by using this cellular model, such as dose-dependent retinoic acid response and effects of the ligands of the other nuclear receptors. We also showed that even with only 2500 cells the luminescent response was detectable. These models take benefit from the well known advantages of the firefly luciferase as reporter gene: detection in intact living cells, sensitivity and handiness.

P12

T.S. Dobmeyer, J.M. Dobmeyer, B. Raffel, M. Rehder, B. Morsches and R.E. Schopf, University of Mainz, Department of Dermatology, Mainz, Germany.

HLA-DR+ EPIDERMAL CELLS STIMULATE THE SKIN-LYMPHOCYTE REACTION IN GREATER EXTENT THAN CD1a+ CELLS

We examined the importance of CD1a+ HLA-DR+ Langerhans cells and CD1a-HLA-Dr+ epidermal cells for the stimulation of allogeneic and autologous T cells. Mononuclear leukocytes isolated from the peripheral blood of 7 healthy individuals and 7 patients with psoriasis were enriched to 96 % by passage over nylon wool columns. Epidermal cells were isolated from the roofs of suction blisters by trypsinization and depletion of CD1a+ and HLA-Dr+ cells by immunobeads. Cell enrichment and depletion were ascertained by FACS analysis. 1×10^5 enriched T cells were incubated for 6 days with 1×10^4 allogeneic and autologous epidermal cells. Cell proliferation was measured by ^{3}H-TdR uptake. We found no significant difference between healthy controls and patients with psoriasis. In both groups depletion of CD1a+ epidermal cells led to decreased stimulation of T cells, both in the allogeneic and autologous systems. Compared to

CD1a+ cells, depletion of HLA-DR+ epidermal cells further significantly diminished T cell proliferation in controls and patients. We conclude that HLA-DR+ epidermal cells are more important than CD1a+ Langerhans cells for stimulation of both allogeneic and autologous T cells both in normal controls and patients with psoriasis. Moreover, our results indicate that there exists a CD1a-HLA-DR+ epidermal cell population capable to stimulate T cells.

P13

J.M. Dobmeyer, T.S. Dobmeyer, B. Raffel, M. Rehder, B. Morsches and R.E. Schopf, University of Mainz, Department of Dermatology, Mainz, Germany.

TNFα PRODUCTION IN THE MIXED EPIDERMAL-LYMPHOCYTE REACTION IN PSORIASIS: INFLUENCE OF HLA-DR+ and CD1a+ STIMULATOR AND CD4+ RESPONDER T CELLS

Autologous epidermal cells are able to stimulate T cells. The aim of our study was to measure TNFα production after depleting stimulator epidermal HLA-DR+ and CD1a+ cells, and enriching CD4+ T responder cells in mixed epidermal-lymphocyte cultures. T cells were enriched to > 96 % CD3+ cells by passage of PBL over nylon wool columns, CD4+ cells were positively selected to 80 % by immunomagnetic beads as checked by FACS analysis. Epidermal cells were isolated by suction blister techniques, CD1a+ Langerhans cells and HLA-DR+ cells were depleted by immunomagnetic beads. 1 x 10^5 CD3+ cells were incubated with 1 x 10^4 epidermal cells in RPMI 1640/10 % AB-serum for 5 days. TFNα was determined in cell culture supernatants by ELISA technique. In healthy individuals the TNFα concentration in the cell cultures was unaffected by depletion of either CD1a+ or HLA-DR+ stimulator cells. By contrast, TNFα concentrations dropped after depletion of both CD1a+ and HLA-DR+ cells in cultures of patients with psoriasis. In cultures enriched for CD4+ responder cells, TNFα production was only marginal in healthy controls, whereas in psoriasis enrichment of CD4+ responder cells led to increased TNFα production. We conclude that TNFα production in the epidermal cell-lymphocyte reaction is abnormal in psoriasis. Both stimulator Langerhans cells and responder CD4+ are important for TNFα production in epidermal-lymphocyte reactions in patients with psoriasis but not in healthy controls.

P14

L. Duteil, C. Queille-Roussel and J. Czernielewski, Centre International de Recherches Dermatologiques (CIRD) GALDERMA, Sophia Antipolis, Valbonne and Centre de Pharmacologie Clinique Appliquée à la Dermatologie (CPCAD), Hôpital Pasteur, Nice, France.

RETINOIC ACID PRETREATMENT EFFECT ON INDUCED SKIN INFLAMMATION

In this study, the effect of 15 days retinoic acid (RA) pretreatment has been assessed in three types of induced skin inflammation: ultraviolet B (UVB) erythema, sodium lauryl sulfate (SLS) skin irritation and nickel contact dermatitis.

Eighteen healthy subjects, amongst which 6 were nickel-positive, were included in the study. Three zones (3x10 cm) were delineated in the middle back of each subject. One zone was treated with RA 0.05% (RETIN-A cream), another with a cream base (SKINBASE) and the third was left untreated. The products were applied once daily for 15 days. At the end of the pretreatment period, the subjects were divided in three groups of 6. Each group was submitted to one type of skin inflammation. In the UVB and SLS tests, four doses of inflammatory stimuli were applied on each tested zone. Quantification of erythema by colorimetry (parameter a*) and measurements of transepidermal water loss (TEWL, integrity index of stratum corneum) were performed before and at the end of pretreatment, and during the evolution of the induced inflammatory reactions.

At the end of the pretreatment period, no signs of irritation (erythema or scaling) were clinically observable. On the other hand, a significant increase of TEWL and a* was detected on RA treated zones, indicating the presence of subclinical erythema and stratum corneum modifications. No statistical differences between pretreatments were detected on SLS induced skin reactions. The UVB erythema had a tendency (N.S) to be increased by the RA pretreatment. Clinical and biophysical evaluations showed a significant ($p<0.05$) exacerbation of nickel contact dermatitis on RA pretreated zones. Thus, in nickel sensitive subjects, a non clinically irritating skin pretreatment with RA may alter the skin barrier function, and hence increase susceptibility to allergens.

P15

B. Farkas, B. Bonnekoh* and G. Mahrle*, Albert Szent-Györgyi Medical University, Department of Dermatology, Szeged, Hungary and * University of Cologne, Department of Dermatology, Cologne, Germany.

REGULATORY EFFECTS OF 1,25-DIHYDROXY-VITAMIN D_3 AND CALCIPOTRIOL ON HUMAN KERATINOCYTE CELL LINE (HACAT)

Recent studies provide evidence that $1,25(OH)_2D_3$ has an effect on the regulation of immune responses on some cells such as human promyelocytic leukaemia cells, normal human monocytes, murine monocyte/macrophage tumor cells and mouse keratinocytes (PAM 212) (Tani, M., Br.J.Dermatol. 1992, 126, 266). Most, if not all of the biological actions (e.g. induction of cell differentiation, inhibition of cell division, modulation of immune functions) of $1,25(OH)_2D_3$ (VD_3) and of calcipotriol (CPT) are believed to be mediated by a high-affinity nuclear receptor (VDR) for the vitamin D hormone.

In the present study the hyperproliferative HaCat cells were examined to determine whether they are targets for VD_3 and for CPT. VD_3 and CPT at 10^{-6} M significantly decreased the level of DNA synthesis, and at 10^{-6}-10^{-8} M the rate of protein synthesis. HaCat cells respond to VD_3 and its synthetic analogue CPT by modulating growth kinetics suggesting the existence of a vitamine D autocrine loop in this cell model to psoriatic keratinocytes. Further investigations are needed to determine the effects of VD_3 and CPT on the regulation of immune response.

P16

J.R. Gibson, Bristol-Myers Squibb, Pharmaceutical Research Institute, Buffalo, N.Y./USA.

A HUMAN MODEL FOR THE HISTOLOGICAL EVALUATION OF DRUG-INDUCED BIOLOGICAL SKIN EFFECTS WHICH MAY BE PERTINENT TO AGING-RELATED SKIN CHANGES, INCLUDING PHOTO-DAMAGE.

To date, most work targeting aging-related skin changes, including photodamage, with a view to drug development in this area has focused on topically applied retinoids, most notably tretinoin. Animal models for predicting possible clinical benefit of agents in this condition are currently unproven for use with non-retinoid compounds and are of less than optimal value in the testing of retinoid and retinoid-like agents and their formulations. Our aim was to explore the use of a human skin model that may indicate biological activity of both retinoid and non-retinoid materials and could be subsequently validated as a predictor of clinical benefit in aging-related skin changes.

The model for discussion involves the use of 20-40 subjects per test who are treated (with/without occlusion, as necessary) on multiple forearm sites for 28 days according to a randomized, double-blind plan with a test agent, tretinoin (positive control), vehicle, a potent corticosteroid and a combination of the corticosteroid and the test agent. Punch biopsies at the end of treatment yield histological data concerning changes in viable epidermal thickness, presence of increased/decreased ground substance and other relevant parameters. Various other evaluations including clinical "pre-atrophy" determination, ultrasound measurements for skin thickness and density, transepidermal water loss, skin impedance and skin elasticity are performed in order to provide additional data which may correlate with histological findings.

Available data show that the results obtained from this model may aid in elucidating the potential for biological skin effects of both retinoid and non-retinoid compounds and in decisions concerning their optimal concentration in, and bioavailability from, various formulations. Refinement and full validation of this model will, of course, be necessary.

P17

C.E.M. Griffiths, L.J. Finkel, M.G. Tranfaglia, T.A. Hamilton and J.J. Voorhees, University of Michigan, Department of Dermatology, Ann Arbor, Mi./USA.

A HUMAN BIOASSAY FOR THE IN VIVO STUDY OF TOPICAL RETINOIC ACID ACTIVITY

An occlusive patch test assay has been developed for assessment of topical retinoid action in human epidermis. Previous work with this assay has demonstrated marked epidermal hyperplasia in normal skin treated with topical all-*trans* retinoic acid (RA) for 4 days under "Saran Wrap" occlusion and similar effects with the local irritant, sodium lauryl sulfate (SLS). Time course, dose response and comparison with SLS, were performed with RA.

At no time, between 1 and 4 days, could the clinical or histologic effects (stratum corneum compaction, epidermal and granular layer thickness, spongiosis, and mitotic index) of 0.1 % and 0.025 % cream formulations of RA be distinguished from each other (n = 10). An "overall response index" of retinoid effects was devised which amalgamates scores for erythema, epidermal thickness and spongiosis on a 0-12 scale. This index was used to generate a 4 day dose response for RA at concentrations from 0.001 % to 0.025 % dissolved in 70 % ethanol/30 % propylene glycol vehicle (n = 30). RA could be successfully differentiated from SLS at 2 days by virtue of its greater ability to increase epidermal thickness, spongiosis and glycosaminoglycan deposition (n = 10).

It appears that although RA and SLS produce similar epidermal histologic changes at 4 days, significant differences at earlier time-points suggest differing mechanisms of action which are probably dependent on receptor binding in the case of RA and independent of receptor binding in the case of SLS. Additionally, this assay is able to provide potency ranking for doses of RA and is of use in furthering our understanding of the cutaneous pharmacology of this agent.

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I. Grillier, W. Bourguet*, B. Sablonniere, D. Manechez, J.Y. Chen**, P. Formstecher and M. Dautrevaux, Faculté de Médecine, Laboratoire de Biochimie Structural, *INSERM U 16, Lille and **Faculté de Médecine, INSERM U 184/LGME, Institut de Chimie Biologique, Strasbourg, France.

STRUCTURE ACTIVITY RELATIONSHIPS OF NOVEL SUBSTITUTED CHALCONE DERIVATIVES

Retinoic acid and other synthetic retinoids are now used as therapeutic agents in the treatment of acute promyelocytic leukemia and also in proliferative skin disorders. Their biological effects are mediated by three nuclear receptors name RARα , β and γ. The identification of synthetic retinoids selective for each RAR subtype would represent a valuable tool. Such retinoids might target a limited number of tissues, thus decreasing their overall toxicity. We have tested the relative affinity of various chalcone derivatives for the three human recombinant receptors produced in infected SF9 cells using baculoviruses expressing RARs.The K_D values of the three overexpressed receptors were in the range 4-6x10^{-9}M. Various chalcone compounds, including the 2'substituted derivatives were obtained as potential ligands for affinity chromatography (BW24, BW24$_{Cu}$, BW25, BW29, BW252). Their relative affinities was tested by competition assays with retinoic acid. CD367 was tested as reference compound displaying Ki values of 7 to 10x10^{-9}M. Most of these compounds showed a relative affinity similar to the one of Ch55 (Ki=0.4 x 10^{-6}M). Introduction of a 2'acetoxy substituent had only a limited effect on affinity, (BW29, Ki=0.7x10^{-6}M) which was sharply reduced by substitution with the bulky 2' benzoxy group (Ki=14x10^{-6}M). None of these compounds has selective binding activity. The 2'3 amino propanoxyl derivative (BW26) displaying a Ki value of 2 to 4x10^{-6}M with a slight specificity towards hRARγ, was used to the design of an affinity matrix. Biological activity of these compounds was also assayed by measuring the induction of HL60 cells differentiation by the NBT reduction test. Whereas the BW24 derivative and the Ch55 were strongly active, the other derivatives were inactive. Transactivational activity of some of these compounds, is now being tested in transfected COS cells.

P19

M.C. Lenoir-Viale, C. Galup, Y.M. Darmon, B. Shroot and B.A. Bernard, Centre International de Recherches Dermatologiques (CIRD) Galderma, Sophia Antipolis, Valbonne, France

A NEW RECONSTRUCTED EPIDERMIS MODEL DERIVED FROM HUMAN HAIR FOLLICLE AND DE-EPIDERMIZED DERMIS

Human epidermis can be reconstructed in vitro by cultivating hair follicles on a dermal equivalent made of collagen type I and living fibroblasts (Lenoir et al.1988, Dev.Biol. 130, 610-620). This was interpreted as a phenotypical transition of cultured outer root sheath cells. To assess the possible role of living fibroblasts in this process, we repeated the experiment by cultivating human hair follicles, but on de-epidermized dermis (Pruniéras et al. 1979, Ann.Chir.Plas. 24, 357-362) .

The cultures were kept immerged for 6 days, and thereafter were elevated to the air-liquid interface. A multilayered and differentiated epidermis was obtained by 15 days. This epidermis presented the characteristic features of normal human epidermis in vivo. A complete basal membrane with numerous hemidesmosomes was seen. The basal cells were well oriented with their main axis perpendicular to the dermo-epidermal junction. Spinous and granular layers could be observed, with numerous keratohyalin granules. Cornified layers laid on top of the cultures. By in situ hybridization, keratin k5 and k10 mRNA was localized in the basal and suprabasal cells respectively, like in normal human epidermis. These results demonstrate that the differentiation pathway of human outer root sheath cells is intrinsically labile and can be shifted towards the interfollicular keratinocyte pattern by air exposure, even in the absence of dermal fibroblasts. The differentiation of reconstructed epidermis is modified by retinoic acid treatment, in a dose-dependent manner. This culture system might thus represent a valuable and promising tool for pharmacological studies on in vitro reconstructed skin.

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D. Manechez, W. Bourguet*, N. Tbarka, J.L. Bernier*, J.P. Henichart*, P. Formstecher and M. Dautrevaux, Faculté de Médecine, Laboratoire de Biochimie Structurale, and *Inserm U16, Lille, France.

INVOLVEMENT OF PROTEIN KINASE C PATHWAY IN RETINOIC ACID-INDUCED TISSUE TRANSGLUTAMINASE EXPRESSION. DIFFERENTIAL RESPONSE INDUCED BY RETINOIC ACID AND SYNTHETIC RETINOIDS.

Cellular differentiation and tissue transglutaminase (tTG), a programmed cell death marker, are both induced by retinoic acid (RA) in various cells as epidermal cells. This effect is likely to be mediated by retinoic acid receptors (RARs). Effects of chalcone retinobenzoic acids, Ch55 and a 4'-hydroxyl derivative, were studied on these two responses in human promyelocytic leukemia cells HL-60 and neuroblastoma cells SK-N-SH. Surprisingly, different dose-response curves were observed between RA and retinobenzoic acids on tTG expression, although those compounds had an identical effect on cellular differentiation. Very low concentrations (10^{-11}M) of retinobenzoic acids induce tTG activity although higher concentrations of RA (10^{-9}M) were necessary to induce the same response. Moreover, at higher concentrations (10^{-7}M), RA induced tTG activity and enzyme concentration was five-fold higher than response induced by retinobenzoic acids. These agents were not partial agonists as shown by competition experiments. A different mechanism of action for RA and retinobenzoic acids effects is likely, involving other transduction pathways than RARs. To test for this hypothesis, 20 μM H7 and 20 μM H8, respectively protein kinase C (PKC) and A (PKA) inhibitors, were used. Interestingly, H7 was able to reduce RA-induced tTG but failed to alter this response when induced by retinobenzoic acids. Finally, synergistic effects were observed when retinobenzoic acids and tetradecanoyl phorbol acetate (TPA), a PKC activator, were associated. Involvement of PKC in RA-induced tTG expression could be a general phenomena in other cells as epidermal cells.

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M. Michel, L. Germain and F. Auger, Hôpital du St.-Sacrement, Laboratoire des Grands Brûlés/LOEX, Université Laval, Quebec, Canada.

PERCUTANEOUS ABSORPTION PROPERTIES OF CULTURED SKIN EQUIVALENT IN VITRO: INFLUENCE OF KERATINOCYTE CONCENTRATION AND CULTURE CONDITIONS

Percutaneous absorption is one of the most important biological function of the skin; skin layers, particularly the stratum corneum, play an important role in skin barrier properties. Skin permeability can be measured in vitro using Franz diffusion cells. Skin from hairless animals is used in most pharmacological studies. However, a large variability in the results is observed.

We produced a living human skin equivalent (SE) in vitro. Keratinocytes were cultured on a fibroblast populated collagen gel (Bell et al., Science 211, 1042, 1981) and formed a stratified multilayer which reached a terminal differentiation state when cultured at an air-liquid interface. A new peripheral anchorage technique for these SE was established in our laboratory (Lopez-Valle et al., Brit.J.Dermatol, in press). This model inhibits collagen contraction and allows SE to be mounted on Franz diffusion chambers. Percutaneous absorption properties of dermal equivalents and SE were measured, using benzoic acid, a molecule with a high permeability index. The results suggest that a decrease in SE permeability was proportional to the presence, the growth and differentiation rate of keratinocytes in culture. In fact, SE cultured at an air-liquid interface were less permeable than immersed cultures. In all cases, histological analysis of the SE revealed a correlation between permeability and stratum corneum thickness. These results confirm that keratinocytes are directly involved in skin permeability, notably differentiated cells, forming the stratum corneum. This was the first attempt to optimize the cultured SE for permeability measurement for pharmacological and cosmetological studies.

P22

H.M. Ockenfels, B. Morsches* and R.E. Schopf*, University of Essen, Department of Dermatology, Essen and *University of Mainz, Department of Dermatology, Mainz, Germany.

CHLOROQUINE, ETHANOL AND INDOMETHACIN AFFECT LYMPHOCYTE PROLIFERATION IN PATIENTS WITH PSORIASIS

A variety of agents including chloroquine and ethanol have been reported to trigger psoriasis. Results about the correlation between the exacerbation of psoriasis and the medication of indomethacin are contradicting. Since immunological mechanisms are considered to be of great importance in the pathogenesis of psoriasis, we compared the effect of different concentrations of chloroquine, ethanol and indomethacin on the in vitro lymphocyte proliferation in 15 healthy control individuals and 15 patients with psoriasis. The lymphocyte proliferation was monitored by the spontaneous and phytohemagglutin (PHA)-induced uptake of ^{3}H-TdR.

Both spontaneous and PHA-driven lymphocyte proliferation were significantly lower in samples from patients with psoriasis. Spontaneous blastogenesis both in control samples and in psoriatic samples remained unaffected by these drugs. In PHA-induced cultures from controls, chloroquine diminished proliferation dependent on the concentration; ethanol reduced the proliferation by more than 50 %. By contrast, ethanol increased the proliferation of lymphocytes from psoriatic patients by 200-300 % and chloroquine increased the proliferation by more than 300 %. Indomethacin did not stimulate the PHA-induced lymphocyte proliferation neither in psoriatic nor in control samples, on the contrary, proliferation was slightly decreased in both groups.

Our data show that in psoriatic samples the lymphocyte transformation is unaffected by indomethacin but abnormally increased by the addition of chloroquine and ethanol, pointing out the possible impact of chloroquine and ethanol on the pathomechanisms of psoriasis. Ethanol and chloroquine influence the activation process of T-lymphocytes of psoriasis patients in vitro, we assume an analogous effect on immunological pathways in vivo.

P23

U. Pfeffer, C. Brigati, N. Ferrari, F. Tosetti, A. Profumo, E. Fecarotta and G. Vidali, Istituto Nazionale per la Ricerca sul Cancro, Molecular Biology Laboratory, Genova, Italy.

SEQUENCE COMPARISON OF THE DNA BINDING DOMAINS OF RAR AND RXR RETINOID RECEPTORS

The action of retinoids is mediated by nuclear receptors, RAR and RXR, which belong to the superfamily of thryoid and steroid hormone receptors. The DNA binding domain of these receptors consists of two zinc fingers (4 Cys type) and is highly conserved within the whole family. Significant amino acid sequence differences are found in a region corresponding to the second knuckle of the first zinc finger which has been shown to be responsible for the hormone response element specificity. The

superfamily thus can be divided into two subfamilies, the progesterone, mineralocorticoid, androgene and glucocorticoid receptor family (knuckle sequence: CGSCKV) and the estrogen, thyroid, vitamin D and retinoic acid receptor family (knuckle sequence: CEGCKG(A)).

Alignment of retinoic acid receptor RAR and RXR amino acid sequences reveals the extreme conservation of the DNA binding domains. The more recently detected RXR subfamily also contains the consensus knuckle sequence CEGCKG. However, the sequence of the first knuckle of the same zinc finger is different: CFVCQ(N) for the RAR subfamily and CAICG for the RXR subfamily. Although the substitutions of phenylalanine by alanine and of valine by isoleucin can be considered conservative ones, these and the substitution of the polar side chain of glutamine by the non polar side chain of glycine might have a functional significance. This is even more likely as the two sequences are conserved even in species as distant as Homo sapiens and Xenopus leavis. It is also possible that the two sequences evolved from a common ancestor early in evolution before the development of the alpha, beta and gamma subtypes of each receptor. As a consequence, species that possess different receptors for the two retinoid pathways (RAR and RXR) but without the subtypes (alpha, beta and gamma) should exist. The alignment of the sequences also shows few other conservative and non conservative amino acid substitutions, the functional significance of which we still ignore.

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S.R. Pinnell and S. Murad, Duke University Medical Center, Durham, NC./USA.

SUPPRESSION OF COLLAGEN SYNTHESIS AND PROLIFERATION OF FIBROBLASTS IN CULTURE BY BENZOIC HYDRAZIDE

In confluent cultures of human skin fibroblasts, collagen synthesis measured as [^{3}H]proline incorporation into collagenase-sensitive protein was specifically inhibited by benzoic hydrazide (BH) in a dose- and time-dependent manner. The maximum inhibition (80 %) occurred in cells treated with 1mM BH for 72 hr. A number of other hydrazides as well as benzamide and ethyl benzoate were inactive, suggesting extremely rigid structural requirements for collagen-suppressing activity. The BH effect was independent of the presence of ascorbic acid and the concentration of serum in culture medium. BH-treated cells regained full capacity to synthesize collagen when cultured for 72 hr in medium devoid of BH. The diminished collagen synthesis in BH-treated cells correlated with a specific reduction in the level of procollagen mRNA (63 %). The little collagen synthesized by BH-treated fibroblasts was predominantly associated with the cell layer and was deficient in hydroxyproline (76 %), indicating inhibition of prolyl hydroxylation in the cell. When added to cell extracts, however, 1 mM BH had no effect on prolyl hydroxylase activity. Taken together, these data indicate that under the culture conditions BH is activated to an inhibitor of prolyl hydroxylase. In addition to suppressing collagen synthesis, BH reversibly inhibited the serum-dependent proliferation of subconfluent cultures without causing cytotoxicity. The antiproliferative effect was dependent on BH concentration between 10 µM and 100 µM, the latter resulting in almost complete cessation of cell proliferation. Under these conditions, phenylacetic and isonicotinic hydrazides had no significant effect on fibroblast proliferation, reflecting their inability to inhibit collagen synthesis. The studies reveal a requirement of collagen for fibroblast proliferation and offer a therapeutic potential for benzoic hydrazide as an antifibrotic agent.

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E.P. Prens, E. Liem, M. Kozel, O. Ijsselmuiden*, R. Benner and T. van Joost*, University Hospital Dijkzigt and Erasmus University Rotterdam, Departments of Dermatology and *Immunology, Rotterdam, The Netherlands.

EFFECTS OF ORAL CYCLOSPORIN A ON THE EXPRESSION OF CYTOKINES IN LESIONAL PSORIATIC SKIN

Cyclosporin A (CsA) has been shown to be efficacious in psoriasis. CsA may theoretically improve psoriasis via inhibition of (T lymphocyte derived) cytokines and of keratinocyte proliferation. The levels of expression of cytokines in cryostat sections from lesional skin of eight patients with recalcitrant plaque-type psoriasis were studied. CsA (Sandimmune^R) was given orally at a dose of 5 mg/kg/day. Skin biopsies were taken from the same psoriatic plaques, before and after 2 weeks of therapy. Specific antibodies to IL-1α, IL-1β, TNF-α, IL-6, IL-8, IFN-γ were used in an indirect immunoperoxidase staining technique. Antibodies to IL-2R, T lymphocytes and some adhesion molecules were also included. The results showed that the expression of IL-1β (but not IL-1α) and, to a lesser extent, of IL-8 were significantly reduced in the epidermis after treatment. The total number of T lymphocytes was significantly reduced in the dermal infiltrate in all CsA-treated patients. However, the CD4/CD8 ratio remained unaltered. As reported by others, IL-2R$^+$ and ICAM-1$^+$ cells were almost completely depleted from the epidermis after two weeks of CsA treatment. These observations stress the role of IL-1β, IL-8 and T lymphocytes in the pathophysiology of psoriasis.

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P.T. Pugliese and J.-C. Farina*, Milmark Research Inc., Bernville, Pa./USA and *F. Hoffmann-La Roche Ltd., Basel, Switzerland.

PANTOTHENIC ACID PRECURSOR IN WOUND HEALING: DOUBLE BLIND ASSESSMENT BY ULTRA-SOUND AND HISTOLOGICAL EXAMINATION OF EXCISED WOUNDS

Pantothenic acid (PA) is a component of Coenzyme A (CoA). CoA is vital in many biochemical reactions but is not transported across cell membranes; pantothenic acid is the mobile species. Dexpanthenol, the alcohol analog of PA, is absorbed by the skin and converted to PA. Fibroblasts cultures incubated with PA, or its derivatives increase ^{3}H-thymidine incorporation, cell proliferation, cell migration, collagen synthesis and intracellular protein synesis; the mechanism is unknown. A double blind study was performed to determine if topical dexpanthenol in a suitable base was able to accelerate wound healing. Fifteen volunteers aged 50 to 63 had four wounds made on the parasacral area by shave biopsy. Randomized wounds were treated once daily for 5 days either with 1) dexpanthenol in a w/o base [BEPANTHEN^R Ointment], 2) the base alone, 3) a commercial product, or 4) normal saline using 0.1 ml of product on each wound. Healing was evaluated by erythema index, surface closure, and wound volume decrease; viscoelastic changes and histological examination of the excised wounds were assessed at 19 days.

Results showed significant differences in the elasticity of the wounds, with the dexpanthenol treated wounds showing the fastest recovery. Histologically, the dexpanthenol treated wounds healed faster, and with less scarring than either the base product or the commercial product. This suggest a major role for PA in the wound repair process directed towards fibroblast activity, problably through a specific regulatory mechanism of collagen and elastin synthesis.

These models take benefit from the well known advantages of the firefly luciferase as reporter gene: detection in intact living cells, sensitivity and handiness.

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J. Reichrath, S. Epple, A. Kerber, G. Unteregger*, H.P. Baum and F.A. Bahmer, University of the Saarland, Department of Dermatology and *Institute of Human Genetics, Homburg, Germany.

VITAMIN D RECEPTOR (VDR) COEXPRESSION WITH MEDIATORS OF LOCAL INFLAMMATION IN PSORIASIS VULGARIS: AN IMMUNOHISTOCHEMICAL DEMONSTRATION BY CONFOCAL LASER SCANNING MICROSCOPY

The skin is a key tissue in both synthesis of the precursor and function of vitamin D3. The most potent metabolite 1.25-dihydroxyvitamin D3 (1.25-D3) acts via binding to a nuclear high-affinity receptor (VDR). 1.25-D3 was shown to block proliferation and to promote differentiation in keratinocytes in vitro. The efficacy of topical application of 1.25-D3 or vitamin D analogues in the therapy of psoriasis vulgaris has been shown recently. Proliferation and differentiation of epidermal cells are regulated by various growth factors, cytokines, hormones and corresponding receptors. A significant increase in VDR expression in keratinocytes and skin immune cells in lesional as compared to nonlesional psoriatic skin was shown applying an immunohistochemical method and the monoclonal antibody 9A7 γ to the VDR. Recently, we demonstrated by saturation analyses according to Scatchard that VDR expression in keratinocytes is modulated by cytokines in vitro. This effect seems to be specific since it was not linearly concordant with the effects of those cytokines on proliferation of keratinocytes. We now focused our efforts to demonstrate by confocal laser scanning microscopy the coexpression of VDR and mediators of local inflammation in psoriasis vulgaris in vivo. Double staining procedures were performed applying the monoclonal antibody 9A7 γ to the VDR and antibodies to CD antigens, cytokines (IL-1, NAP-1/IL-8, TNF-α, interferon γ) and corresponding receptors. Our findings indicate that confocal laser scanning microscopy is a promising tool to analyse the coexpression of hormones, cytokines, growth factors and corresponding receptors. The results suggest that local 1.25-D3 concentration, VDR expression and cytokines might be of high importance in the connectivity between skin immune cells and keratinocytes in psoriasis vulgaris and under various other pathological conditions in vivo.

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A. Rolland, N. Wagner, A. Chatelus, B. Shroot and H. Schaefer, Centre International de Recherches Dermatologiques (CIRD) GALDERMA, Sophia Antipolis, Valbonne, France.

POLYMERIC MICROSPHERES AS A NOVEL TOPICAL SITE-SPECIFIC DRUG DELIVERY SYSTEM FOR TARGETING A NAPHTHOIC ACID DERIVATIVE, ADAPALENE, TO THE PILO-SEBACEOUS UNIT

In order to improve the therapeutic index of adapalene (CD 271), a new drug under development for the treatment of acne (1), site-specific drug delivery to the hair follicles using 50:50 poly (D,L) lactic-co-glycolic acid microspheres (PLGA microspheres) as particulate carriers was investigated in vitro and in vivo.

PLGA microspheres (mean diameters: 1, 5 and 20 μm) containing 1% of adapalene were prepared by a solvent evaporation technique.

After application of aqueous gels containing 10% adapalene-loaded PLGA microspheres (i.e. 0.1% w/w adapalene, final concentration) to hairless rat and human skin in vitro for 35 and 300 minutes (static diffusion cell), cryosections of skin pieces were observed by fluorescence and scanning electron microscopy. The 5 μm microspheres were specifically targeted to the follicular ducts and did not penetrate the stratum corneum, whereas the 1 μm microspheres randomly distributed into the stratum corneum and hair follicles. The largest microparticles (20 μm) remained on the stratum corneum surface.

An aqueous gel containing 10% of adapalene-loaded microspheres was not irritating in a rabbit skin irritancy test. A dose-related comedolytic activity of topical formulations of adapalene-loaded microspheres was observed in the rhino mouse model (2).

The in vitro release of adapalene from the microspheres in artificial sebum at 37°C was much faster than the in vivo sebum excretion in humans (8 days), with a half-life of about 60 minutes.

After topical application of an aqueous gel containing adapalene-loaded PLGA microspheres (5 μm) to human volunteers, site-specific drug delivery was further evidenced by follicular biopsy.

Drug follicular targeting using polymeric microspheres (3) may represent a promising and valuable therapeutic approach for pathologies associated with pilo-sebaceous structures.

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A. Rolland, G. Demichelis and B. Shroot, Centre International de Recherches Dermatologiques (CIRD) GALDERMA, Sophia Antipolis, Valbonne, France.

IN VITRO RELEASE OF ADAPALENE FROM DIFFERENT TOPICAL DRUG DELIVERY SYSTEMS

There is a need for rapid and reliable in vitro methods for assessing drug release from topical dosage forms. These tests should allow accurate comparisons of drug release profiles from experimental topical formulations and also constitute a standardized methodology for assuring batch-to-batch uniformity (1, 2).

The release of a new drug developed for the treatment of acne (3), adapalene (CD 271) from various topical drug delivery systems was analysed in vitro using an automated flow-through diffusion cell (4). The influence of receptor fluid polarity, synthetic membrane characteristics, occlusion and formulation composition, on adapalene release was evaluated by calculation of the apparent release constant and lag-time from the release profiles.

The release rate of adapalene from an anionic oil-in-water

emulsion and an aqueous gel was independent of the amount of tested formulation. It was, however, highly dependent on the type of formulation (lotion, aqueous or hydroalcoholic gel, anionic or non-ionic oil-in-water cream, polymeric microspheres). Adapalene release from the different formulations increased proportionally to the receptor fluid lipophilicity (isopropyl myristate >n-octanol >aqueous surfactant solution). The characteristics of the synthetic membrane also influenced adapalene release profiles and drug pharmaceutical availability was greatly enhanced under occlusion for drug delivery systems containing volatile solvents.

The in vitro automated release test proposed in the present study, with a membrane-receptor phase combination and a standard procedure adapted to the drug and dosage form physico-chemical characteristics, represents a valuable and reproducible method for screening prototype topical drug delivery systems and for a batch-to-batch control in the manufacturing process.

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A. Rolland and B. Shroot, Centre International de Recherches Dermatologiques (CIRD) GALDERMA, Sophia Antipolis, Valbonne, France

PREPARATION AND EVALUATION OF DERMATOLOGICAL FORMULATIONS BASED ON INCLUSION OF TRETINOIN IN β-CYCLODEXTRIN AND HYDROXYPROPYL-β-CYCLODEXTRIN

Retinoids, particularly tretinoin (all-trans-retinoic acid), represent a major class of drugs in dermatology for the topical treatment of several diseases such as acne, psoriasis, ichthyosis, actinic keratoses. In order to improve the solubility and stability of tretinoin in aqueous topical dosage forms and concomitantly reduce its skin irritation potency whilst maintaining its therapeutic efficacy, inclusion complexes of tretinoin in either β-cyclodextrin or hydroxypropyl-β-cyclodextrin (1,2) were prepared as described by Shroot et al. (3).

Tretinoin solubility in water was increased by inclusion in β-cyclodextrin and hydroxypropyl-β-cyclodextrin by about 400 and 5000 times, respectively, and a further augmentation was obtained by using the tretinoin triethanolamine salt.

Aqueous solutions containing inclusion complexes of tretinoin with β-cyclodextrin or hydroxypropyl-β-cyclodextrin, jellified with Carbopol (tretinoin final concentration: 0.025%), were stable for 2 months at 45°C and for 2 years at 20°C.

These gels were less irritant than commercial formulations of tretinoin in a rabbit skin irritancy test.

Although the profiles of in vitro tretinoin release from the cyclodextrin drug delivery systems were different from those of commercial formulations, in vitro drug penetration kinetics through hairless rat or human skin were found to be similar.

An aqueous gel containing tretinoin/β-cyclodextrin inclusion complex presented a comedolytic activity at least equivalent to that of a commercial hydroalcoholic gel (Aberel 0.025%) in the rhino mouse model (4).

This aqueous gel was also as effective as the commercial formulation in the topical treatment of patients with acne vulgaris and was better tolerated with in particular reduced irritation.

Cyclodextrins represent a promising advance in dermatology as they enhance significantly the solubility of lipophilic drugs such as retinoids in water and thus permit the use of totally aqueous formulations. They may also protect drugs from oxidation, photo-decomposition and improve their therapeutic index by either reducing toxic side-effects or modifying drug bioavailability.

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T. Rosenbach, B.M. Czarnetzki and W.F. Greenlee, UKRV, Free University Berlin, Department of Dermatology, Clinical Immunology and Asthma, Germany and Purdue University, Department of Pharmacology and Toxicology, West Lafayette, In./USA.

DIOXIN-INDUCED ALTERATIONS ON INOSITOL PHOSPHATE FORMATION IN SCC-12F KERATINOCYTES

The hydrolysis of membrane phosphoinositides is an important signal transduction pathway coupled to cell surface receptors and is suggested to play a role in cell proliferation and differentiation. In the present investigation, the environmental contaminant 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) was studied for its effect on the inositol phosphate pathway in the human keratinocyte cell line SCC-12F. TCDD did not act directly on this pathway to alter inositol phosphate metabolism. Using bradykinin as external activator, pretreatment of SCC-12F cells with 10 nM TCDD for 48 h resulted in an attenuated formation of inositol 1, 4, 5-trisphosphate as compared to vehicle controls (52% reduction). In contrast, TCDD pretreatment yielded an enhanced response (35% increase in inositol monophosphates as compared to vehicle-treated controls) in the presence of the calcium ionophore A23187 or the combination of 20 nM NaF and 10 μM $AlCl_3$ which is known to form AlF_4^-, a putative GTP mimicking agent. These results indicate that TCDD pretreatment can alter the responsiveness of the inositol phosphate signal transduction pathway to both external effectors and intracellular modulators of inositol phosphate formation. Modulation of the inositol phosphate - mediated signal transduction might contribute to TCDD - induced alterations in patterns of growth and differentiation in keratinocytes.

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R.E. Schopf, P. Benes, R. Benz, B. Morsches and Lothar Färber* University of Mainz, Department of Dermatology, Mainz and *Sandoz AG, Nürnberg, Germany

CYCLOSPORIN A OR ETRETINATE TREATMENT NORMALIZE THE DECREASED BINDING OF cAMP TO PROTEIN KINASE A IN ERYTHROCYTE MEMBRANES OF PATIENTS WITH PSORIASIS

During a therapeutic study with etretinate compared to cyclosporin A in psoriasis the effects of these agents on the binding of cAMP to the regulatory subunit RI of protein kinase-A in erythrocyte membranes were investigated by determining the UV-light catalyzed incorporation of tritiated azido-cAMP into protein kinase-A of isolated erythrocyte membranes. Erythrocyte membranes from 10 healthy individuals served as controls. The evaluation was done by Scatchard plots. The method allowed the calculation of the maximal binding capacity and also of the dissociation constant Kd. In 10 psoriatics treated with etretinate and 21 psoriatics treated with cyclosporin A the binding capacity of the protein kinase for cAMP was definitely decreased and returned to normal after treatment for 10 weeks. Over this period there was a significant correlation between the psoriasis area-and-severity index (PASI) and the maximal binding capacity. The Kd values, however, remained unchanged before and after treatment. These results found in erythrocytes with a survival time of 120 days indicate a non-competitive inhibition of the cAMP binding rather than a genetic defect. The inhibitor seems to disappear during effective treatment of psoriasis either with etretinate or cyclosporin A.

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J. Sitzmann, B. Algermissen, B.M. Czarnetzki* and P. LeMotte F. Hoffmann-La Roche Ltd., Department of Dermatology, Basel, Switzerland and UKRV, Free University Berlin, Department of Dermatology, Berlin, Germany.

EXPRESSION OF PSORIASIN mRNA IN SEVERAL HUMAN SKIN DISEASES

Psoriasin is a small protein with a potential Ca-binding site but with as yet no known function. It was recently found to be expressed at high levels in keratinocytes from psoriatic plaques but only weakly in keratinocytes from normal skin (J. Invest. Dermatol. 97:701, 1991). We have therefore investigated, by in situ hybridisation, the mRNA distribution of psoriasin in five additional skin diseases: mycosis fungoides, atopic dermatitis, lichen sclerosus et atrophicus, M. Darier and ichthyosis vulgaris.

Riboprobe templates were generated by PCR using self priming oligonucleotides spanning 150 bp of the 3'-end of the published gene sequence flanked by T7- and T3-promotors. In situ hybridisation was performed by standard procedures using [^{35}S]-labelled antisense riboprobes and sections made from routinely formalin fixed and paraffin embedded clinical biopsy material. A similarly designed riboprobe derived from the human cytokeratin 1 gene (K1) served as positive control and sense probes of both templates as negative controls.

Consistent with published results, we observed strong expression of psoriasin mRNA in the epidermis of psoriatic plaques. Furthermore, compared to K1-expression, strong to moderate signals were found in mycosis fungoides, atopic dermatitis, M. Darier and, in some cases, in lichen sclerosus et atrophicus. Expression was much weaker in involved psoriatic skin and not detectable in ichthyosis vulgaris and normal skin. In all cases, psoriasin expression correlated with the existence of subepidermal perivascular cellular infiltrates. In general, signals were clearly suprabasal with an increased expression towards the cornified layer.

These results show that "psoriasin" is not restricted to psoriasis, but that it can also be found in other inflammatory skin diseases. Although its particular role awaits elucidation, psoriasin may be important in the molecular mechanisms underlying the pathogenesis of inflammatory skin diseases.

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V. Stapor, A. Langner, H. Verjans*, M. Mol*, T.P. Chorzelski and J.R. Elzerman*, Warsaw Medical Academy, Warsaw, Poland and *Solvay-Duphar, Clinical Research Department, Weesp, The Netherlands.

DOES TOPICAL APPLICATION OF 1,25-DIHYDROXY-VITAMIN D3 NORMALIZE EPIDERMAL DIFFERENTIATION IN PSORIASIS?

29 patients suffering from chronic plaque psoriasis were treated with ointments containing 3 µg/g 1,25(OH)2D3 (calcitriol) or placebo in a within-patient, left-right, comparative, double-blind, randomized study. The ointments were applied twice daily to symmetrical, bilateral, severe psoriatic plaques for 6 weeks or until clearance of one of the side, whichever was the shortest.

For histological and immunological investigation, skin punch-biopies were taken from the center of each chosen psoriatic plaques before and after the treatment. Skin samples were divided into two fragments. One of them underwent standard histological hematoxylin-eosin staining and the second one was preserved for direct or indirect immunological evaluation using monoclonal antibodies CD 15, CD 16, CD 36, CD 1a, Ki 67.

Clinical results revealed complete clearance or considerable improvement in 72.5 % of calcitriol treated sides and in 31 % of placebo. Local and systemic tolerance and safety of twice daily 3 µg/g calcitriol ointment was satisfactory.

Histological evaluations before and after the treatment clearly showed better results under influence of 1,25-dihydroxyvitamin D3 than placebo, however very good and good results were obtained on both sides of the body, but epidermal normalization was histologically more evident in calcitriol ointment treated plaques.

Immunopathological study was performed in small group of patients. Nevertheless strongly positive pemphigus-like reaction in all biopsies before treatment (CD 36), negative staining with monoclonal antibody (m.a.) CD 1a, strongly positive reaction with m.a. CD 15 and CD 16 and normalization of these pictures with calcitriol therapy suggest its essential influence on epidermal proliferation and differentiation.

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F. Vecchini, J. Magdalou*, B. Shroot and B.A. Bernard, Centre International de Recherches Dermatologiques, Sophia Antipolis, Valbonne and *Centre du Médicament, U.R.A. CNRS 597, Faculté des Sciences Pharmaceutiques et Biologiques, Nancy, France.

IDENTIFICATION AND MODULATION BY RETINOIC ACID OF DRUG METABOLIZING ENZYMES PRESENT IN CULTURED HUMAN SKIN

Although enzymatic activities in the skin can be a key factor for the toxicity of topically applied drugs, little is kown about the xenobiotic metabolizing enzymes present in human keratinocytes. Recently, we have detected both phase I and Phase II drug metabolizing activities in an in vitro reconstituted epidermis (M.-A. Pham et al., J.Invest.Dermatol. 94, 749-752, 1990). Using western blots and a chemiluminescent detection procedure, we now identified, in normal human keratinocytes grown in culture, i) phase I enzymes, namely cytochrome P450 1A1 (60 kDa) and NADPH-reductase (78 kDa), and ii) phase II enzymes, namely UDP-glucuronosyltransferase (64 kDa), and the pi form of gluthathion-S-transferase (23 kDa). With the exception of GSTpi,

expression of these enzymes was not only inducible by 3-methylcholanthrene and dimethylbenz(a)anthracene, but also by retinoic acid.

To better understand the regulation of cytochrome P450 1A1 expression in human keratinocytes, we transfected into these cells CAT constructs containing all or parts of the regulatory region of the 1A1 gene, cloned ahead of the CAT gene (Hines et al., Carcinogenesis 9, 1599-1605, 1988). By functionally scanning this regulatory region in the presence of different inducers, we confirmed that the previously identified xenobiotic responsive elements (XRE) were active in keratinocytes.

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M. Verschoore, A. Langner*, M. Wolska, M.*, S. Jablonska* and J. Czernielewski, Centre International de Recherches Dermatologiques (CIRD) Galderma, Sophia Antipolis, Valbonne, France and *Klinika Dermatologiczna A.M., ULK Koszykowa 82a, Warsaw, Poland

VEHICLE CONTROLLED STUDY OF CD 271 LOTION IN THE TOPICAL TREATMENT OF ACNE VULGARIS

CD 271 is a synthetic, naphthoic acid derivative. It is a powerful modulator of epidermal differentiation with a retinoid-like activity.

This double blind randomized study compared the efficacy and safety of CD 271 0.1% lotion to its vehicle base alone. Two parallel groups of male acne patients were treated twice daily for 8 weeks with CD 271 0.1% lotion (15 patients) or vehicle (15 patients) on their facial acne. Efficacy was assessed by changes in individual lesion counts. Skin reactions were scored from 0 to 3 for erythema, dryness, pruritus and burning at week 1, week 2, week 4 and week 8 of treatment. Twenty-nine cases completed the study and the single drop-out was due to an infections disease not related to treatment.

Analysis of efficacy results showed that at the end of the treatment period :

- Non inflammatory lesions (open + closed comedones) were significantly more reduced ($p<0.01$) with CD 271 0.1% lotion (66% reduction) than with vehicle (30%).
-Inflammatory lesions (papules + pustules + nodules + cysts) were also significantly more reduced ($p<0.01$) with CD 271 0.1% lotion (35% reduction) than with vehicle (11%).
-Reduction of total lesion counts was significantly greater with CD 271 0.1% lotion than with vehicle ($p<0.01$) (59% and 25% reduction respectively).

Erythema, dryness and burning were generally mild and clinically acceptable with CD 271 0.1% lotion, although these effects were significantly higher than with vehicle ($p<0.01$). Intensity of pruritus was significantly more pronounced with CD 271 0.1% lotion than with vehicle at week 8 only, however, this side effect was never severe.

No CD 271 could be detected in blood plasma at the end of the 2 month treatment period (detection limit = 1ng/ml).

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J.P. Viallet, E. Ruberte*, Ph. Kastner*, A. Krust* and D. Dhouailly, Université Joseph Fourier, Laboratoire de Biologie de la Différenciation Epithéliale, CERMO, Grenoble and *Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'INSERM, Strasbourg, France.

RETINOIC ACID-INDUCED GLANDULAR METAPLASIA IN MOUSE SKIN AND RARs EXPRESSION

Using in situ hybridization, the expression of the nuclear retinoic acid receptors, RAR α, β, and γ, and of the cellular retinoic acid binding protein I (CRABP I) has been established during normal mouse skin morphogenesis, as well as after 48 h of retinoic acid (16.5×10^{-6} M) treatment of upper lip skin of 13.5-day mouse embryos in vitro.

The transcripts of RAR α and of RAR γ genes are abundant in dermal cells at the two precise stages at which these cells are known to elicit epidermal placode formation (12.5 days) and follicle morphogenesis (13.5 days). In epidermal cells, the distribution of RAR γ transcripts increases in parallel with the differentiation of the hair follicle. It should be noted that the CRABP gene seems not to be expressed at a noticeable level until the completion of skin morphogenesis (15.5 days). The RAR β signal is barely above background at all these stages. In contrast, the addition of RA provokes a significant up-regulation of the RAR β gene in the dermis, followed by the initiation of an alteration of hair vibrissa development, leading to an exocrine-type gland morphogenesis.

These results suggest a role for retinoic acid and for its receptors in the epidermal-dermal interactions leading to the differentiation of cutaneous appendages, while the CRABP I appears to act in sequestering retinoic acid in skin regions which are not longer involved in morphogenetic processes.